PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/53, 9/06, C12Q 1/00, 1/26, B09C 1/10, C12P 13/00, C12N 1/21, 1/20 // (C12N 1/20, C12R 1:01)

A1

(11) International Publication Number: **WO 97/03201**

(43) International Publication Date: 30 January 1997 (30.01.97)

(21) International Application Number: PCT/GB96/01629

(22) International Filing Date: 8 July 1996 (08.07.96)

(30) Priority Data:

9514138.8	11 July 1995 (11.07.95)	GB
9607220.2	4 April 1996 (04.04.96)	GB

(71) Applicant *(for all designated States except US)*: THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation & Research Agency, DRA Farnborough, Hampshire GU14 6TD (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: NICKLIN, Stephen [GB/GB]; 6 Ridgelands, Penhurst Road, Bidborough, Kent TN3 0XE (GB). BINKS, Peter, Roland [GB/GB]; 16 Seaview Avenue, Irby, Wirral, Merseyside L61 3UE (GB). BRUCE, Neil, Charles [GB/GB]; Trinity Hall, Cambridge CB2 1TJ (GB). FRENCH, Christopher, Edward [GB/GB]; University of Cambridge, Institute of Biotechnology, Tennis Court Road, Cambridge CB2 1TJ (GB).

(74) Agent: BOWDERY, Anthony, Oliver; D/IPR (DERA) Formalities, Poplar 2, MOD (PE) Abbey Wood #19, P.O. Box 702, Bristol BS12 7DU (GB).

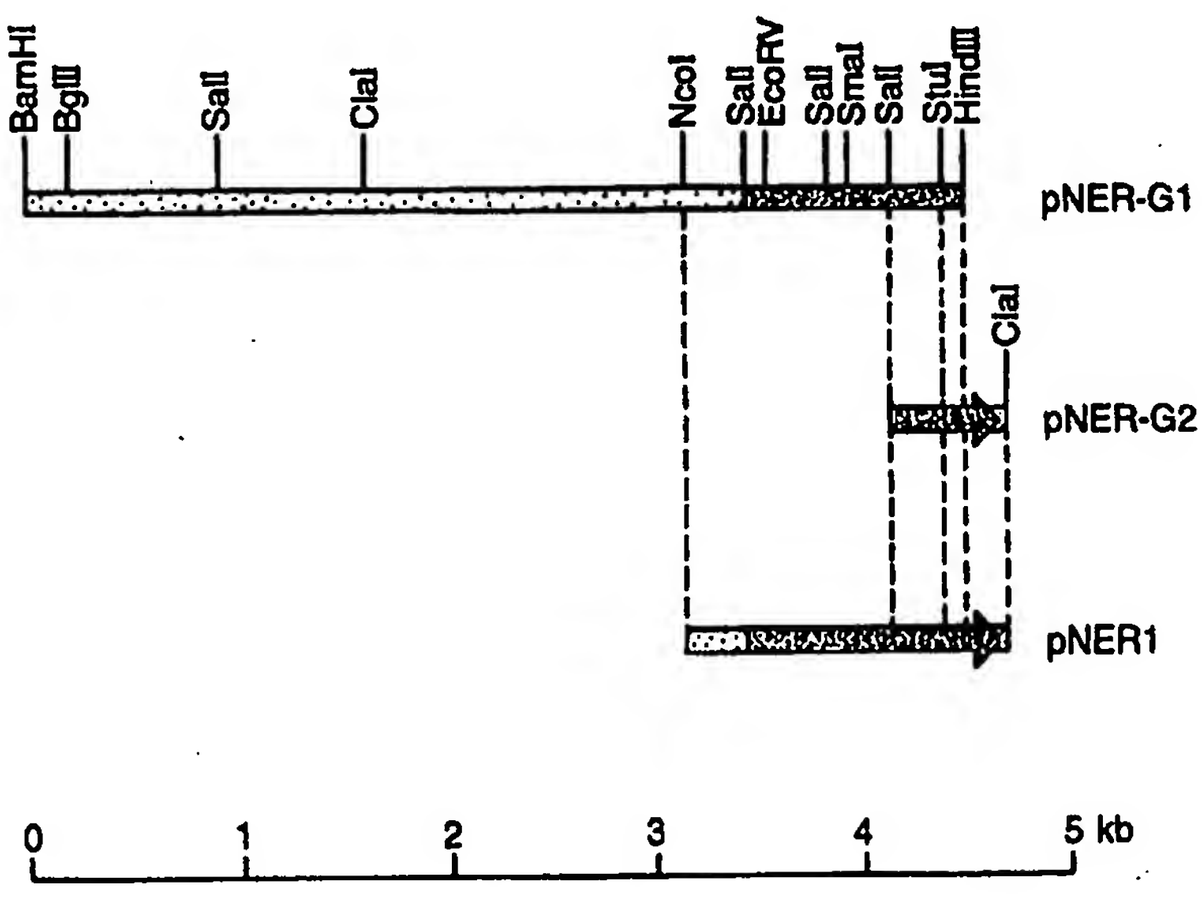
(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DETECTION AND BIODEGRADATION OF EXPLOSIVES



(57) Abstract

An enzyme capable of catalyzing the removal of nitrite from pentaerythritol tetranitrate (PETN) is provided. The enzyme (known as PETN reductase enzyme) is produced by culturing a novel strain of the Enterobacter cloacae bacterium isolated from nature. The strain designated PB2 has been deposited as NCIMB 40718. The amino acid sequence of the enzyme and the genetic sequence which encodes for this enzyme have also been determined. A PETN reductase enzyme encoded by the on gene is provided. A method for producing PETN reductase enzyme in large quantities and methods of bioremediation using the enzyme so produced are also provided. Additionally there is provided a method of detecting the presence of PETN in a sample together with a biosensor for use in such a method.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

Detection and Biodegradation of Explosives

This invention relates to the field of explosives detection and biodegradation and in particular to a novel enzyme, to the amino acid sequence for the enzyme, to the gene which encodes the enzyme and to methods of producing the enzyme recombinantly. The invention further relates to a method of using the enzyme to catalyze the aerobic biodegredation of pentaerythritol tetranitrate (hereinafter referred to by the commonly used abbreviation PETN) and to a method and apparatus for the detection of PETN using the enzyme.

The novel enzyme of this invention has also been demonstrated to liberate nitrite from glycerol trinitrate (GTN) and from ethylene glycol dinitrate (EGDN) which are further examples of nitrate ester species. The enzyme is referred to as a PETN reductase.

Nitrate esters, though apparently extremely rare in nature, are produced in significant quantities by the chemical industry and comprise, for example, an important class of energetic materials having applicability as explosives and propellants. PETN itself has a diverse range of applications including as an explosive in blasting caps and detonators and in pharmaceutical compositions as the active ingredient of long-acting, slow onset coronary vasodilators for the prevention of angina attacks. The manufacture, handling and disposal of PETN can all lead to the contamination of the environment with PETN. There are concerns regarding the environmental fate of nitrate esters due to their relative recalcitrance and there therefore exists a need for a means of removing such contaminants from the environment without producing other undesirable pollutants. There is also an urgent requirement for a better method of detecting PETN as the currently proposed analytical systems rely mostly on use of bulky and sophisticated pieces of equipment such as gas chromatograms or mass spectrometers and/or require specially trained laboratory technicians for their application.

It is an aim of this invention to provide an enzyme which is capable of catalyzing the biodegradation of PETN and which may be employed in a bioremediation system for the environmental decontamination of the PETN pollutant. It is a further aim to provide an enzyme which is useful for PETN detection systems.

According to a first aspect of the invention therefore there is provided a PETN reductase enzyme which has the characteristics that, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH hereinafter), it:-

1) catalyses the removal of nitrite from PETN; and

2) has reductase activity specifically at the nitrate ester linkage of PETN.

The enzyme is further characterised by the fact that it has a pH optimum of 6.5 and that it has a M_r of about 40,000 Daltons as determined by gel filtration. The subunit M_r, as estimated by SDS-PAGE (polyacrylamide gel electrophoresis), was also 40 000. These results suggest that PETN reductase is a monomer of M_r approximately 40,000.

The PETN reductase enzyme of this invention is characterised by the essential features described above but may be further identified by additional characteristics such as its pH optimum, catalytic activity, thermal stability or molecular weight. Details of such further characteristics are given in Example 3 below but it must be stressed that these characteristics are variable to a degree depending upon the conditions under which the microorganism producing the enzyme is grown and upon the degree of purification of the crude product. Variations of this kind will be well understood by those skilled in the art.

The purified enzyme is visibly yellow and shows a visible absorption spectrum characteristic of an oxidised flavoprotein. The flavin was liberated from PETN reductase by boiling followed by removal of denatured protein by centifugation, indicating that the flavin is not covalently bound. In two thin layer chromatography systems the liberated flavin comigrated with authentic flavin mononucleotide (FMN) and not with flavin adenine dinucleotide (FAD). Flavin standards subjected to the protocol used to liberate the flavin

from PETN reductase showed no change; in particular, FAD was not hydrolysed to FMN. These results indicate therefore that the enzyme is a monomeric flavoprotein which binds FMN non-covalently.

The amino acid sequence of the enzyme and the encoding genetic nucleotide sequence therefor have been established. According to a second aspect of the present invention therefore, there is provided a PETN reductase enzyme having the amino acid sequence shown in Figure 4 or a derivative thereof. By derivative herein is meant a version of the enzyme sequence of Figure 4 containing insertions, deletions and/or substitutions of the amino acid sequence such that the functionality of the enzyme is retained.

Based on the evidence of the amino acid sequence it is possible to state that the enzyme is a member of the family of α/β-barrel flavoprotein oxidoreductases.

In a third aspect, the present invention provides the gene (designated *onr* (for organic nitrate ester reductase)) which encodes the PETN reductase enzyme or a derivative thereof. By derivative of the gene is meant herein homologues of the gene having a coding sequence which is at least 70% identical to the *onr* gene, involving any and all single or multiple nucleotide additions, deletions and/or substitutions thereto.

According to a fourth aspect of the invention there is provided a recombinant DNA molecule including the nucleotide sequence of the *onr* gene or a nucleotide sequence which is at least 70% identical to the nucleotide sequence of the *onr* gene. The nucleotide sequence of the *onr* gene as determined herein is set out in Figure 3.

In a fifth aspect the present invention provides an enzyme comprising substantially the transcribed product of the *onr* gene.

In a further aspect the present invention provides the PETN reductase enzyme which is encoded by the *onr* gene.

It will be readily apparent to those skilled in the art that once the sequence of the genetic material which encodes for PETN reductase enzyme in the *E. cloacae* PB2 microorganism has been identified in accordance with this present invention, it will be possible according to well known techniques to transform a suitable host cell with the *onr* gene (or a derivative thereof) such that the host cell produces (expresses) the recombinant gene product ie. PETN reductase. This may be done by any suitable method such as splicing the gene into a vector and transforming said host cell with that vector.

Accordingly the present invention therefore also provides a recombinant DNA vector containing the *onr* gene or a derivative thereof and a host cell transformed with the *onr* gene or a derivative thereof. This transformation may be performed using such a vector.

The manner in which such vectors and recombinant DNA may be produced will be readily apparent to those skilled in the art given that the methods applicable, including the choice of a suitable host cell and of suitable promoters are generally well understood in the field.

Among host cells which might be used for the purpose, it may be appropriate to use *E. cloacae* PB2 cells transformed with additional copies of the *onr* gene.

Using the host cell transformed with the *onr* gene or a derivative thereof and spliced behind a suitable promoter of the host cell, it will be possible to generate PETN reductase enzyme in industrially useful quantities, again by techniques which are well known in the art. There is also provided therefore, a method for the production of PETN reductase enzyme or a derivative thereof which comprises the steps of:

transforming a host cell with the *onr* gene or a derivative thereof;

growing the host cell under conditions appropriate for expression of the said *onr* gene product, and

extracting the *onr* gene product from the growing medium or from the host cells after disruption thereof.

Most conveniently the production process is run in a continuous or semi-continuous manner to continuously extract the enzyme produced from the growth medium for the host cell, employing techniques which are well understood in the art. If the host cell produces the enzyme intracellularly, disruption of the cells in the culture will be necessary to extract the enzyme product. Suitable host cells may be provided by either prokaryotic or eukaryotic organisms.

The action of the novel reductase enzyme of the present invention is to catalyze the reduction of PETN into pentaerythritol tri- and di-nitrates by attack at the nitrate ester linkage of PETN. The ability to specifically attack the nitrate ester linkage of PETN is a distinctive feature of this PETN reductase enzyme that is not possessed by commercially available reductases. A description of the mode of identification of the reaction products of PETN is set out in Example 2 below.

The ability of the novel PETN reductase enzyme to catalyse the removal of nitrite from PETN in the presence of NADPH allows the enzyme to be used in the detection of PETN. According to a yet further aspect of the invention therefore, there is provided a method of detecting the presence of PETN in a sample which comprises subjecting the sample to the PETN reductase enzyme of this invention in the presence of NADPH and under conditions to permit reaction of any PETN present in the sample and detecting the occurrence of such reaction. Conveniently, detection of the reaction would be by means of a bioluminescence test using either bacterial or firefly luciferase, or by a colorimetric or amperometric method as is well known in the art.

In a further aspect, therefore, the present invention also provides a biosensor for the detection of PETN in a sample which comprises means for contacting the sample with a PETN reductase enzyme in the presence of NADPH and means for detecting the

occurrence of a reaction, catalyzed by the enzyme, of PETN when PETN is present in the sample. The means for detecting the occurrence of a reaction may conveniently comprise a bioluminescent transducer or an amperometric transducer. Such sensors can be used as the basis for highly convenient portable detectors for checking luggage, clothing etc. for traces of PETN.

The methods of making biosensors that rely on amperometric or bioluminescent changes in the test reaction are well known in the art. For example, UK Patent Application Publication No. 2,231,332A (NRDC) describes such methods and their use in biosensors, the contents of which in relation thereof is hereby incorporated by reference. Any of such methods may be of use in detecting the occurrence of the PETN degrading reaction and hence in the detection of PETN.

Typically, in the case of a bioluminescent biosensor, luciferase is used. This enzyme is responsible for the light-emitting reaction of luminous bacteria and catalyzes the reaction of molecular oxygen with reduced flavin and aliphatic aldehyde to form long-lived intermediates whose slow breakdown provides energy to give light emission with reasonably high quantum yield. In coupling the PETN reductase to such a bioluminescent system, the $NADP^+$ generated through the activity of the PETN reductase is detected by the oxidation of an alcohol such as decanol, octanol or hexanol, to its corresponding aldehyde in the presence of an alcohol dehydrogenase. The aldehyde is then detected by reaction with reduced flavin ($FMNH_2$) in the presence of oxygen together with luciferase to catalyse the reaction. The intensity of the emitted light provides a measure of the $NADP^+$ converted to NADPH which in turn provides a measure of the PETN concentration present.

It is anticipated that using such a scheme, sensitivity to PETN will be in the range 0.1 nmol - 0.1μmol.

A convenient colorimetic detection procedure uses a Griess reagent which generates a characteristic purple colouration in the presence of nitrite. A standard curve

comprises the step of inoculating the environment with a sample of the bacterial isolate of *Enterobacter cloacae* designated PB2 and allowing the isolate to consume the PETN present in the environment. The environment concerned may be, for example, a waste stream of material containing PETN originating from the destruction of an explosives charge containing PETN or a sample of PETN-contaminated earth or other material. In the former case bioremedial treatment may be conveniently carried out in a reactor vessel, whereas in the latter instance the isolate may be introduced directly into the environment by inoculating the contaminated soil with it. Other appropriate methods of effecting treatment will be readily apparent to those skilled in the art.

The bacterium identified above will only produce the enzymic activity to which the present invention refers when it is cultured on PETN as the nitrogen source. Further characteristics of the deposited bacterium PB2 are listed below.

Gram stain		-ve
Spores		-ve
Motility		+ve
Growth	37°C	+ve
	41°C	+ve
	45°C	+ve
Catalase		+ve
Oxidase		-ve
Fermentative in glucose OF		+ve
Colony morphology:		Round, regular, entire, smooth, glossy cream, low convex, semi-translucent, 1mm in diameter.

Rapid Test (API): 24 hours at 30°C

β galactosidase	+
Arginine dihydrolase	+

Lysine decarboxylase	-
Ornithine decarboxylase	+
Citrate utilisation	+
H_2S production	-
Urease	-
Tryptophan deaminase	-
Indole production	-
Voges Proskauer	+
Gelatinase	-
Acid production from:-	
Glucose	+
Mannitol	+
Inositol	-
Sorbitol	+
Rhamnose	+
Sucrose	+
Melibiose	+
Amygdalin	+
L (+) Arabinose	+
Cytochrome oxidase	-

Further Tests, 30^0C, 7 days

Methyl Red (37^0C)	-
Acid from Raffinose	+
Acid from α-Methyl-glucoside	+
Acid from Adonitol	+
Gelatin	+

PETN reductase can be produced by culturing *E. cloacae* on PETN as a nitrogen source. When grown on NH_4NO_3 the activity can be induced by the addition of PETN.

comprises the step of inoculating the environment with a sample of the bacterial isolate of *Enterobacter cloacae* designated PB2 and allowing the isolate to consume the PETN present in the environment. The environment concerned may be, for example, a waste stream of material containing PETN originating from the destruction of an explosives charge containing PETN or a sample of PETN-contaminated earth or other material. In the former case bioremedial treatment may be conveniently carried out in a reactor vessel, whereas in the latter instance the isolate may be introduced directly into the environment by inoculating the contaminated soil with it. Other appropriate methods of effecting treatment will be readily apparent to those skilled in the art.

The bacterium identified above will only produce the enzymic activity to which the present invention refers when it is cultured on PETN as the nitrogen source. Further characteristics of the deposited bacterium PB2 are listed below.

Gram stain		-ve
Spores		-ve
Motility		+ve
Growth	37°C	+ve
	41°C	+ve
	45°C	+ve
Catalase		+ve
Oxidase		-ve
Fermentative in glucose OF		+ve
Colony morphology:		Round, regular, entire, smooth, glossy cream, low convex, semi-translucent, 1mm in diameter.

Rapid Test (API): 24 hours at 30°C

β galactosidase	+
Arginine dihydrolase	+

Lysine decarboxylase	-
Ornithine decarboxylase	+
Citrate utilisation	+
H_2S production	-
Urease	-
Tryptophan deaminase	-
Indole production	-
Voges Proskauer	+
Gelatinase	-
Acid production from:-	
Glucose	+
Mannitol	+
Inositol	-
Sorbitol	+
Rhamnose	+
Sucrose	+
Melibiose	+
Amygdalin	+
L (+) Arabinose	+
Cytochrome oxidase	-

Further Tests, 30^0C, 7 days

Methyl Red (37^0C)	-
Acid from Raffinose	+
Acid from α-Methyl-glucoside	+
Acid from Adonitol	+
Gelatin	+

PETN reductase can be produced by culturing *E. cloacae* on PETN as a nitrogen source. When grown on NH_4NO_3 the activity can be induced by the addition of PETN.

However, this is not the case when grown on NH_4Cl. Cultivation is preferably anoxic at any usual temperature, eg within 20 to 40^{0}C range, preferably 25 to 30^{0}C. To obtain PETN reductase the cells can be disrupted in any conventional way. Preferably, a cell free extract is made. The enzyme is then recovered from the extract.

Instead of the precise starting organism deposited, a mutant thereof, eg derived by gamma-ray irradiation or the use of a chemical mutant, induction by culture on another medium etc. or a transconjugant thereof with another bacterium or an artificially produced variant can be used. The ability of any such organism to give the enzymic activity can be readily determined by the skilled person.

In a further manner of use of the previously described activity of the PETN reductase of this invention, there is provided a method for producing pentaerythritol di- and tri-nitrates which may have useful pharmaceutical activity (biotransformation) by reacting PETN with the PETN reductase enzyme of the present invention, in the presence of NADPH.

The invention will now be further described with reference to the following figures of which:-

Figure 1 shows an alignment of the DNA inserts in pNER-G1, pNER-G2 and pNER 1;

Figure 2 demonstrates in schematic manner the stategy used for sequencing *onr* and flanking DNA;

Figure 3 shows the nucleotide sequence of *onr* and the deduced amino acid sequence of PETN reductase;

Figure 4 sets out the amino acid sequence per se; and

Figure 5 is a PETN reductase assay standard curve for PETN-contaminated soils

and to the following examples which further illustrate the invention but are not to be regarded as restrictive thereof.

EXAMPLE 1

Preparation of the enzyme activity from the bacterial strain *Enterobacter cloacae* PB2

1.Materials and Methods

Enterobacter cloacae PB2 was isolated using techniques standard in the art, from samples collected from a natural source by enrichment with PETN as the nitrogen source.

E. cloacae was grown in 1 litre of defined medium consisting of 10 mM potassium phosphate buffer, pH 7.3, containing 0.25 mM $MgS0_4$, 5 mM glucose, 5 mM succinate, 10 mM glycerol, 2mM NH_4NO_3, PETN (10 mM) and trace elements (as described by Pfennig and Lippert, Arch. Microbiology. 1966, 55, 726-739.) supplemented with $CaCl_2.2H_20$ (100mg/l).

Flasks were incubated at 180 r.p.m in a shaking incubator at 30°C. For bulk preparation of bacteria, 1 litre of seed culture was aseptically added to a 75 litre culture vessel, containing 50 litres of sterile medium. The bulk cultures were incubated at 30°C, stirred at 150 r.p.m. with sterile aeration to maintain dissolved oxygen at 10% saturation.

Cell free extracts were prepared from cells grown in the above manner. Cells were harvested from a 75 litre bulk culture, using continuous flow centrifugation (Sorval TZ-28 rotor, Sorval RC-5C centrifuge). Those obtained from a smaller volume culture were then

pelleted by spinning at 10,000g for 15 min at 4^{0}C in a Sorval RC-SC centrifuge fitted with a GS-3 rotor. These pelleted cells were resuspended in 2 ml of bis-Tris propane buffer (pH 7), per gram wet cell weight. Cells were disrupted by sonication in an MSE Soniprep (Fisons, Instruments, FSA Ltd.) using 6 x 12 μm bursts of 15 seconds, alternated with 30 seconds of cooling in melted ice. Cell debris and unbroken cells were removed by centrifugation at 20,000g for 20 min at 4^{0}C in a Sorval RC-5C centrifuge using a SS-34 rotor, to give clarified cell free extract.

2 Chemicals

PETN was of the highest purity and other chemicals were of analytical grade, and unless stated otherwise, were obtained from BDH Ltd. (Poole, U.K), Sigma Chemical Company Ltd. (Poole, U.K) or Aldrich (Gillingham, U.K).

3 Assays

PETN reductase

PETN reductase activity was determined by monitoring the disappearance of PETN by HPLC in 50 mM bis-Tris propane buffer (pH 7), containing PETN (47 μM, final concentration), 40 μl enzyme and NADPH (0.2 mM, fmal concentration) in a final volume of 1 ml.

Alternatively, PETN degradation was also followed by monitoring the release of nitrite using Greiss reagent (Rosenblatt, Burrows, Mitchell and Parmer. 1991: "Organic Explosives and Related Compounds" in The Handbook of Environmental Chemistry 3 (G), edited by O.Hutzinger, Springer-Verlag). The assay was carried out as described above and terminated by the addition of ferricyanide (0.5 mM, final concentration) and phenazine methosulphate (0.2 mM, final concentration). Sulphanilic acid (0.6 mM, final concentration) was added and left to stand for 15 min. N-1-naphthylethylenediamine (0.4

mM, final concentration) was then added and after 5 min the colour which developed was measured spectrophotometrically at 540 nm. The unit enzyme activity is defined as the amount of enzyme necessary to release 1 µmol of nitrite per min at 30^0C.

The degradation of PETN could also be determined by monitoring the oxidation of NADPH at 340 nm.

Protein

Protein was routinely assayed by the Coomassie dye-binding method of Bradford (Anal. Biochem. (1976) 72, 248-254) using commercially available reagent and Bovine Serum Albumin standard (Pierce Ltd.- obtained through Life Science Labs Ltd., Luton). An aliquate (20 µl) of sample containing 0.2-1 mg protein/ml was added to 1 ml of reagent and the reaction allowed to develop for 5 min at room temperature prior to reading the absorbance at 595 nm against a blank of buffer (20 µl) plus reagent (1 ml). Comparison to a standard curve of standard values (0-1 mg/ml) allowed calculation of the protein concentration in the sample.

Gel filtration standards

The following enzymes were used as molecular weight markers in gel filtration experiments: Bovine Serum albumin, Ovalbumin, Chymotrypsin and Ribonuclease A (molecular weights 67,000, 43,000, 25,000, 13,700 Daltons respectively).

EXAMPLE 2

Purification of PETN reductase

To the crude extract, obtained from 30g wet weight cells, enough ammonium sulphate was added to achieve 50% saturation. After stirring at 4^0C for 5 min the resulting precipitate was removed by centrifugation at 20,000g for 20 min. Ammonium sulphate was added to the resulting supenatent to achieve 90% saturation. The resulting precipitate was collected by centrifugation at 4^0C and redissolved in 4ml of 50 mM bis-Tris propane buffer (pH 7). The sample was desalted using a PD-10 column packed with Sephadex G-25M (Pharmacia) and concentrated to 8ml by ultrafiltration using an Amicon stirred ultrafiltration chamber equipped with a Diaflo type YM-3 membrane filter that retained proteins with molecular weights greater than 3,000 Daltons. The fast protein liquid chromatography (FPLC) system was used in combination with a Q-sepharose column to further purify the sample. The FPLC system consisted of two LKB P500 pumps (Pharmacia) combined with a model LCC-500 PLUS gradient controller (Pharmacia), a Rheodyne injection valve, a single path UV monitor (Pharmacia) and an LKB 2212 HELIRAC fraction collector. FPLC was carried out at room temperature, but the fractions collected were cooled on ice. The concentrate was applied to the Q-sepharose column (1 x 7 cm) which had previously been equilibrated with 50 mM bis-Tris propane (pH 8.5) at room temperature. The column, was washed extensively with buffer until no further absorbance at 280 nm was detected in the eluent, whence the reductase was eluted with 10 mM NaCl. Fractions (14ml) were collected at a flow rate of 1 ml/min, desalted and concentrated by ultrafiltration as previously described. The sample was then applied to a Mimetic Orange 2 affinity chromatography column (8 x 20 mm, Affinity Chromatography Ltd.) that had previously been equilibrated with 50 mM bis-Tris propane (pH 7) at 4^0C. The column was washed with 2 column volumes of buffer. The third column volume contained the reductase activity which was collected.

Results

The specific activity of the PETN reductase was measured after the various stages of purification described above. The resulting data is set out in Table 1, from which it may be seen that in a cell free extract of *E cloacae* PB2 grown on PETN as a nitrogen source, PETN reductase was present at a specific activity of 0.025 unit/mg protein while ultimately it was purified 182 fold.

Table I

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery of activity (%)	Purification factor (-fold)
Crude extract	40	361.6	9.04	0.025	100	-
Ammonium sulphate fractionation	8	31.4	3.47	0.110	39	4
Q-Sepharose ion exchange chromatography	10.5	15	0.83	0.720	9	29
Mimetic Orange 2 affinity chromatography	16	0.16	0.728	4.550	8	182

EXAMPLE 3

Characteristics of PETN reductase

pH optimum

Purified PETN reductase (50 µl, 0.5 µg protein) was incubated for 4 min at 30^{0}C with 47 µM PETN in a range of buffers: 50 mM bis-Tris propane (pH 6.5, 7, 7.5 and 8), 50 mM 2-[N-morpholino]ethanesulphonic acid (MES) (pH 5.5, 6, 6.5) and 50 mM bis-Tris (pH 6.2, 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8). The concentration of nitrite was then measured using Greiss reagent.

PETN reductase displayed a pH optimum of 6.5.

K_m of enzyme

The K_m of PETN reductase was above the maximum solubility of PETN (47 µM).

Molecular weight detemination

The molecular weight of the native enzyme was determined by the method of Andrews (Biochem. J. (1964) **91**, 222-223). Measurements were carried out on a Superose 6 HR 10/30 column (1 x 30 cm). Purified PETN reductase (10 µg) was mixed with marker proteins and added to the column. The column was eluted with bis-Tris propane buffer (pH 7) and 0.4 ml fractions collected. The elution volume of PETN reductase corresponded to a molecular weight of 40,000 Daltons.

Molecular weight determination was also performed using SDS-PAGE. The purified PETN reductase ran as a distinct major band corresponding to a molecular weight of 42,000 Daltons. This similar value to that of the native enzyme implies that any effect of

detergent/protein interactions in determining the molecular weight of the enzyme were minimal.

EXAMPLE 4

Cloning and sequencing of the structural gene *onr* encoding PETN reductase.

The structural gene encoding PETN reductase, designated *onr* (for organic nitrate ester reductase) was cloned by the use of degenerate oligonucleotide probes based on the N-terminal amino acid sequence of the purified protein.

To obtain the N-terminal amino acid sequence of PETN reductase, purified enzyme (4.0 μg) was transferred from an SDS/polyacrylamide gel to a poly(vinylidene difluoride) membrane (ProBlott; Applied Biosystems, Foster City, CA, USA) using the Pharmacia PhastTransfer semi-dry blotting apparatus (Pharmacia Biotech, St. Albans, UK). The transfer buffer consisted of 25 mM tris(hydroxymethyl)aminomethane, 190 mM glycine, 10% w/v methanol. Transfer was with a current of 25 mA for 30 min. The N-terminal amino acid sequence was determined by automated Edman degradation by the Protein and Nucleic Acid Chemistry Facility, Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge.

Based on elements of the amino acid sequence, the following oligonucleotide probes were designed:

1) ACTTT(G/C)AG(G/C)GG(G/C)GTGAA(G/C)AGTTTTTC(G/C)GC

2) GT(G/C)AG(G/C)GG(GC)GCCATGAA(G/C)AC(G/C)CGGTT

Oligonucleotides were synthesized by the Protein and Nucleic Acid Chemistry Facility, Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge.

Genomic DNA was prepared from *E. cloacae* PB2 as described by Ausubel *et al.* (Ausubel, F. M., Brent, R., Kingston, R. E. *et al.* (1994) Current Protocols in Molecular Biology, John Wiley and Sons, New York). Genomic DNA was digested to completion with various combinations of restriction endonucleases obtained from Boehringer-Mannheim (Lewes, E. Sussex, UK), Gibco/BRL (Paisley, Scotland, UK) and Promega (Southampton, Hants., UK). DNA fragments were separated by agarose gel electrophoresis using 0.8% (w/v) agarose gels and TAE buffer (Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), and transferred to nylon membranes (Hybond N+, Amersham, Little Chalfont, Bucks., UK) by alkaline capillary blotting according to the manufacturer's protocol.

The oligonucleotide probes described above were end-labelled using [γ-^{32}P]ATP (Dupont/NEN) and T4 Polynucleotide Kinase (Promega). Blots were prehybridized for 2 h at 42^{0}C in a hybridization buffer consisting of 5 x SSC (where 1 x SSC is 0.15 M NaCl/0.015 M sodium citrate) with 5 x Denhardt's solution (where 1 x Denhardt's is 0.02% Ficoll 400/0.02% polyvinyl-pyrrolidone/0.02% bovine serum albumin) and 0.02 mg/ml sonicated denatured salmon sperm DNA. Labelled oligonucleotide probes were then added at 1 pmol/ml and allowed to hybridize overnight at the same temperature. Blots were washed four times for 15 min in 0.5 x SSC/0.1% (w/v) SDS at 45^{0}C and exposed to Fuji RX100 X-ray film.

Both oligonucleotide probes were found to hybridize to the same region of genomic DNA. A *Bam* III*/Hin* dIII fragment of 4.5 kb was selected for cloning. The vector used was pBluescript SK+ (Stratagene, Cambridge, UK). Genomic DNA was digested with *Bam* HI and *Hin* dIII and DNA fragments of 4 to 5 kb were eluted from agarose gels using the US Bioclean system (United States Biochemical Corporation, obtained through Amersham). Vector DNA was digested with the same enzymes, and vector and insert DNA were ligated using T4 DNA Ligase (Promega or Gibco/BRL). Electrocompetent *Escherichia coli* JMIO9 (obtained from Promega) was transformed with the ligation

mixture by electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Hemel Hempstead, Herts., UK). DNA from ampicillin-resistant transformants was transferred to Hybond-N+ membrane (Amersham) by colony blotting according to the manufacturer's protocol. Blots were screened using radiolabelled oligonucleotide probes as described above.

One clone, designated pNER-G1, was found to contain a 4.5 kb insert which hybridized to both probes. DNA sequencing demonstrated the presence of DNA encoding the N-terminal sequence of PETN reductase but showed that the *Hin* dIII site fell within the open reading frame so that the 3' end of the gene was missing (Figure 1). A further oligonucleotide probe, GACGCCGTGGCCTTTGGCCGTGAC, was designed based on the DNA sequence near the *Hin* DIII site, and was used to screen various restriction digests of genomic DNA as described above in order to locate the missing region. Hybridization with this probe was at 50^{0}C and blots were washed at 55^{0}C, twice for 15 min with 1 x SSC/0.1 % (w/v) SDS and twice for 15 min with 0.2 x SSC/0.1 % (w/v) SDS. A *Sal I/Cla* I fragment of 0.5 kb was selected for cloning and was cloned as described above. This clone was designated pNER-G2. The regions of pNER-G 1 and pNER-G2 bearing the *onr* gene were ligated together in pBluescript SK+ to create an *Nco* 1/*Cla* I insert of 1.5 kb bearing the entire *onr* gene. This construct was designated pNER1 (Figure 1). Cell extracts from overnight cultures of *E. coli* JM109/pNER1 grown in a rich medium were prepared and assayed for PETN reductase activity. PETN reductase appeared to form approximately 15% of soluble protein.

Fragments of DNA from the region encoding PETN reductase were subcloned from pNER-G1, pNER-G2 and pNER1 in the vector pBluescript SK+ and sequenced using primers based on vector sequence. The sequencing strategy used is shown in Figure 2. DNA for sequencing was prepared using the Qiagen Plasmid Mini Kit (Qiagen Ltd., Dorking, Surrey, UK). DNA sequencing was peformed by the DNA Sequencing Facility, Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge using a Pharmacia ALF DNA sequencer (Pharmacia Biotech, St. Albans, UK). The nucleotide sequence of the *onr* gene and the deduced amino acid sequence of PETN reductase are shown in Figure 3.

Considering Figure 1 further, it will be noted that all inserts are in the multi-cloning site of the vector pBlueseript SK+ (Stratagene). The dark arrow indicates the position of the *onr* gene encoding PETN reductase. The insert of pNER1 was prepared by ligating together the *Nco* I/*Stu* I region of pNER-Gl with the *Stu* I/*Cla* I region of pNER-G2. In pNER1 the *Nco* 1/*Cla* I fragment is inserted between the *Bam* HI and *Cla* I sites of the multi-cloning site of pBluescript SK+.

With particular reference to Figure 2, subclones were prepared in pBluescript SK+ and sequenced using primers based on vector sequence. Arrows indicate sequencing reactions. The dark arrow indicates the coding region. The positions of restriction sites used in sequencing are numbered, with 1 being the A of the ATG initiating the coding region.

In Figure 3, nucleotides are numbered with 1 being the A of the ATG initiating the coding region. A putative ribosome-binding site is indicated. It should be noted that the N-terminal amino acid sequence of PETN reductase shows that in *E. cloacae* PB2 the initiating methionine is removed. The amino acid sequence is shown independently in Figure 4.

Alternative ways of obtaining and cloning the gene will also be clear to the skilled person having knowledge of the gene sequence. For example suitable restriction enzymes may be employed to "cut" the DNA at restriction sites flanking the gene. Amplification of the gene may also be performed using PCR techniques employing suitably chosen primers flanking the gene.

EXAMPLE 5

Identfication of whole cell degradation products

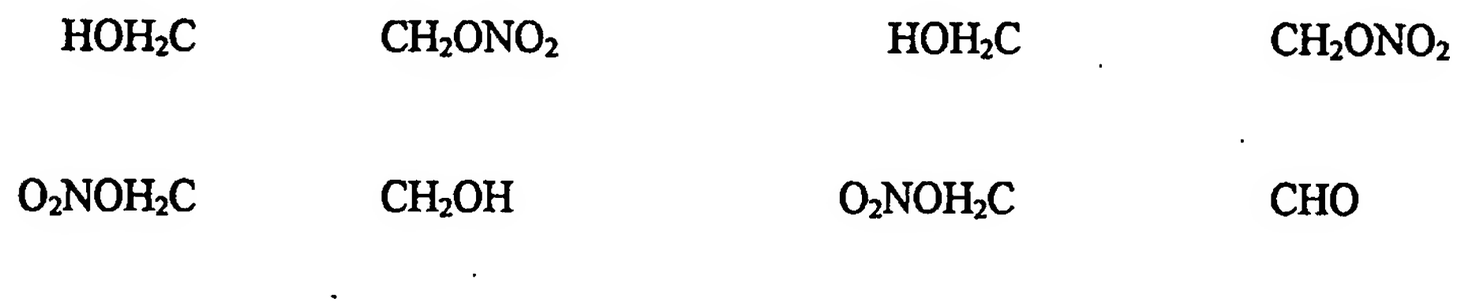
Ethyl acetate extracts of supernatants from cultures grown with PETN contained PETN and three metabolites (as judged by HPLC). The metabolites were separated from

PETN by TLC and found to have lower R_f values (0.64, 0.53 and 0.23) than PETN (R_f 0.81), indicating that the unknown metabolites were more polar than PETN.

The identity of the unknown metabolites was investigated using electron impact (EI) mass spectrometry. The EI mass spectrum of metabolite 1 revealed a molecular ion of m/z 227 which is consistent with the empirical formula $C_5H_{10}N_2O_8$. The mass spectrum contained a fragment ion at m/z 209. It is thought that this metabolite is pentaerythritol dinitrate (2,2-*bis*[(nitrooxy)methyl]-1,3-propanediol), a denitration product of PETN.

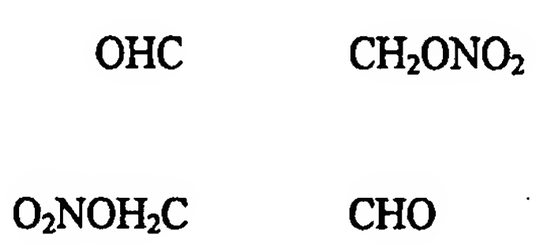
The EI mass spectrum of metabolite 2 revealed a molecular ion of m/z 224 which corresponds to the empirical formula $C_5H_8N_2O_8$. The mass spectrum contained fragment ions at m/z 207, 170, 141, 99, 76, 74, 158, 46 and 44. This is believed to represent 3-hydroxy-2,2-*bis*[(nitrooxy)methyl]propanal.

The EI mass spectrum of metabolite 3 revealed a molecular ion of m/z 222 which corresponds to the empirical formula $C_5H_6N_2O_8$. The mass spectrum contained fragment ions at m/z 208, 177 and 91. This is believed to represent 2,2-bis[(nitrooxy)methyl]-propanedial. The proposed structures for these metabolites are as follows:-



Metabolite 1

Metabolite 2



Metabolite 3

The identity of Metabolite 1 was further investigated using ^{1}H NMR at 400mhz. The ^{1}H NMR spectrum of pure PETN gave a single peak at 4.77 ppm which corresponds to the four equivalent methylene groups in PETN, whilst the ^{1}H NMR spectrum of the unknown metabolite gave two separate peaks at 4.77 and 2.08 ppm. It is believed that the peak at 2.08 ppm corresponds to the methylene groups attached to the hydroxyl group in the proposed structural formula.

The (EI) mass spectroscopy and ^{1}H NMR analysis of the unknown metabolites formed during PETN degradation by the bacterium suggest that at least two nitrogen atoms were used per PETN molecule; these metabolites provide no evidence for the removal of a third nitrate group.

EXAMPLE 6

Identification of the reaction products of PETN reductase

Following treatment of PETN with purified PETN reductase and NADPH, an ethyl acetate extract of the reaction mixture contained PETN and two metabolites (as judged by HPLC). The metabolites were separated from PETN by TLC and found to have lower R_f values (0.78 and 0.64) than PETN (R_f 0.8 1).

The identity of the unknown metabolites was investigated using (EI) mass spectrometry. The EI mass spectrum of metabolite A revealed a molecular ion of m/z 271 which corresponds to the empirical formula $C_5H_9N_3O_{10}$. The mass spectrum contained fragment ions at m/z 239 and 207. This is believed to represent pentaerythritol trinitrate. The EI mass spectrum and R_f value of metabolite B were identical to those of Metabolite 1 isolated from culture supenatant (see Example 2). This metabolite was identified as pentaerythritol dinitrate (PEDN). It therefore appears that PETN reductase reductively liberates nitrite from PETN to form pentaerythritol trinitrate and then PEDN, which is not a

substrate for this enzyme. The presence of aldehydes formed from these alcohols in culture supernatants (Example 2) suggests subsequent dehydrogenase activity by another enzyme.

The specific activity of PETN reductase in crude extract was approximately 0.32 U/mg, equivalent to 9.7 mmol PETN(g soluble protein)$^{-1}h^{-1}$ assuming complete conversion of PETN to PEDN. This is probably sufficient to account for the observed specific degradation rate of PETN by growing cells of 1.03 mmol PETN (g protein)$^{-1}h^{-1}$.

EXAMPLE 7

Use of PETN reductase enzyme as a colorimetric detector for PETN

1.Methodology

This relies on the action of PETN reductase on PETN to produce nitrite which is then monitored by use of a Griess reagent. There was added to a series of uncontaminated soil samples (John Innes No. 2, 1g in each case) placed in clean glass boiling tubes, known concentrations of PETN (from 1 to 500µg) in HPLC acetone. Each of the samples was mixed thoroughly, placed in a vacuum oven and dried for 1 hour at room temperature.

These deliberately contaminated soil samples together with other soil samples (1g) collected from ground which had been contaminated with either PETN or RDX/TNT mixtures were then each treated with 1.5 ml acetone per g of soil sample and shaken for 10 min. The soil was then allowed to settle under gravity after which 25 µl aliquots of the liquid were taken and added to 5ml Pyrex tubes containing a mixture of 12.8 µl of 7 mg/ml PETN reductase, 2µl of 20 mM NADPH (enzyme cofactor) and 220.2 µl of 50mM phosphate buffered saline. Each sample was mixed and then incubated for 10 min at room temperature. 200 µl of 1% sulphanilamide in 2.5% hydrochloric acid was then added to each tube (this forms a diazonium ion with nitrite) and incubated for a further 2 min prior to

the addition of 40 µl of 0.5% N-(1-naphthyl)-ethylenediamine in water resulting in the generation of a purple-coloured product where PETN is present.

200 µl of each sample was taken and placed in a microtitre plate and the absorbance of the sample then measured on a Ceres 900 UV HDi reader. From those samples derived from soil specimens having known PETN concentrations a standard curve of absorbance against PETN concentration in soil could be plotted (Figure 5). Using this curve, optical densities obtained from samples of contaminated soils with unknown PETN content can be used to give an indication of the level of PETN contamination present within each sample.

Table 2 gives data obtained with a series of samples as indicated. These show that the assay described responds specifically to the levels of PETN in soil samples, that it does not give false positives in the absence of PETN or the presence of other explosives (RDX/TNT) and that it is dependent on the presence of the PETN reductase enzyme.

Table 2

Sample characteristic	Sample No.	Absorbance at 540 nm	Amount of PETN/µg/g soil
no enzyme, 100 µg spiked soil	1	0.125	0
	2	0.156	0
uncontaminated soil	3	0.137	0
	4	0.162	0
	5	0.151	0
TNT/RDX contaminated soil	6	0.148	0
	7	0.131	0
	8	0.195	0
PETN contaminated soils	9	1.229	505
	10	0.482	95
	11	0.764	200

PETN	12	2.124	1200
contaminated	13	0.963	420
soils	14	0.805	280

4

Claims

1. A PETN reductase enzyme characterised in that:

(1) it catalyses the removal of nitrite from PETN; and

(2) it has reductase activity specifically at the nitrate ester linkage of PETN.

2. A PETN reductase enzyme according to claim 1 further characterised by one or more of the following features:-

(3) it has a pH optimum of 6.5; and

(4) it has a native molecular weight of about 40,000 Daltons, as determined by gel filtration chromatography.

3. An enzyme having the amino acid sequence shown in Figure 4 or a derivative thereof.

4. The gene (designated *onr*) which encodes the enzyme, or a derivative thereof.

5. A DNA molecule including the nucleotide sequence of the *onr* gene as shown in Figure 3 or a derivative of that sequence.

6. An enzyme comprising substantially the transcribed product of the *onr* gene.

7. PETN reductase enzyme which is encoded by the gene as claimed in claim 4.

8. A recombinant DNA vector containing the *onr* gene or a derivative thereof.

9. A host cell transformed with the *onr* gene or a derivative thereof.

10. A host cell transformed with a DNA molecule as claimed in claim 5.

11. A host cell transformed with a vector as claimed in claim 8.

12. PETN reductase enzyme which is recombinantly produced.

13. A method for the production of PETN reductase enzyme or a derivative thereof which comprises the steps of:

transforming host cells with the *onr* gene or a derivative thereof;

suitably culturing said transformed host cells; and

extracting the enzyme from the growth medium or from the host cells after disruption thereof.

14. A method as claimed in claim 13 where the host cell is transformed by means of a recombinant DNA vector as claimed in claim 8.

15. A method as claimed in claim 13 or claim 14 where the host cell is grown on a continuous basis with the enzyme being continuously extracted from the growth medium.

16. A method as claimed in claim 13 or claim 14 where the host cells in the culture are disrupted after an appropriate period of growth in order to extract the enzyme therefrom.

17. A method of detecting PETN in a sample, comprising subjecting the sample to a reaction involving the removal of nitrite from PETN, the reaction being carried out in the presence of NADPH and a PETN reductase enzyme according to either claim 1 or claim 2, until NADP and nitrite are produced, and detecting the occurrence of said reaction.

18. A method of detecting PETN in a sample, comprising subjecting the sample to a reaction involving the removal of nitrite from PETN, the reaction being carried out in the

presence of NADPH and a PETN reductase enzyme according to any one of claims 3, 6, 7 or 12, until NADP and nitrite are produced, and detecting the occurrence of said reaction.

19. A method according to claim 17 or claim 18 wherein the NADP liberated in the reaction is detected.

20. A method according to claim 19 wherein NADP is detected by a bioluminescence test.

21. A method according to claim 20 wherein the bioluminescence test uses either bacterial or firefly luminescence.

22. A method according to claim 19 wherein NADP is detected amperometrically.

23. A method according to claim 17 or claim 18 wherein the nitrite liberated in the reaction is detected colorimetrically.

24. A method according to claim 23 wherein Griess Reagent is used for colorimetric detection of nitrite.

25. A biosensor for the detection of PETN in a sample which comprises means for contacting the sample with a PETN reductase enzyme in the presence of NADPH and means for detecting the occurrence of a reaction, catalysed by the enzyme, of PETN when PETN is present in the sample.

26. A biosensor for the detection of GTN and/or EDGN in a sample which comprises means for contacting the sample with a PETN reductase enzyme in the presence of NADPH and means for detecting the occurrence of a reaction, catalyzed by the enzyme, of GTN and/or EDGN when either or both is present in the sample.

27. A biosensor according to claim 25 or claim 26 wherein the means for detecting the occurrence of a reaction comprises a bioluminescent or an amperometric transducer or a colorimetric change.

28. A method for the bioremedial treatment of an environment which is contaminated by PETN, GTN and/or EGDN comprising the steps of adding to the environment a quantity of PETN reductase enzyme according to any of claims 3, 6, 7 or 12 and maintaining the mixture under conditions appropriate for degradation of the contaminant by the enzyme so as to allow the PETN, GTN and/or EGDN present in the material to be consumed.

29. A method according to claim 28 wherein the material is a waste stream containing PETN, GTN and/or EGDN.

30. A method according to claim 28 wherein the material is a soil or ground sample contaminated with PETN, GTN and/or EGDN.

31. A method according to any one of claims 28 to 30 wherein the PETN reductase enzyme is produced according to the method as claimed in any one of claims 13 to 16.

32. An *Enterobacter cloacae* bacterial strain referred to as "PB2" and deposited as NCIMB 40718, and mutants and variants thereof capable of producing enzymic activity which degrades PETN in the presence of NADPH.

33. A process of producing an enzyme according to claim 1, which comprises culturing the *Enterobacter cloacae* sp NCIMB 40718 according to claim 32, or a mutant or variant thereof, in the presence of PETN as nitrogen source, at a temperature of from 20 to 40^{0}C, disrupting the cells and recovering the enzyme from the disrupted cells.

34. A method for the bioremedial treatment of a PETN contaminated environment comprising the step of inoculating the environment with a sample of the bacterial isolate of

Enterobacter cloacae as defined in claim 32 and allowing the isolate to consume the PETN present in the environment.

35. A method according to claim 34 wherein the environment is a waste stream containing PETN.

36. A method according to claim 34 wherein the environment is a soil or ground sample contaminated with PETN.

37. A method for producing pentaerythritol di- and tri-nitrates comprising reacting PETN with the PETN reductase enzyme of either claim 1 or claim 2 or of any of claims 3, 6, 7 or 12.

Fig.1.

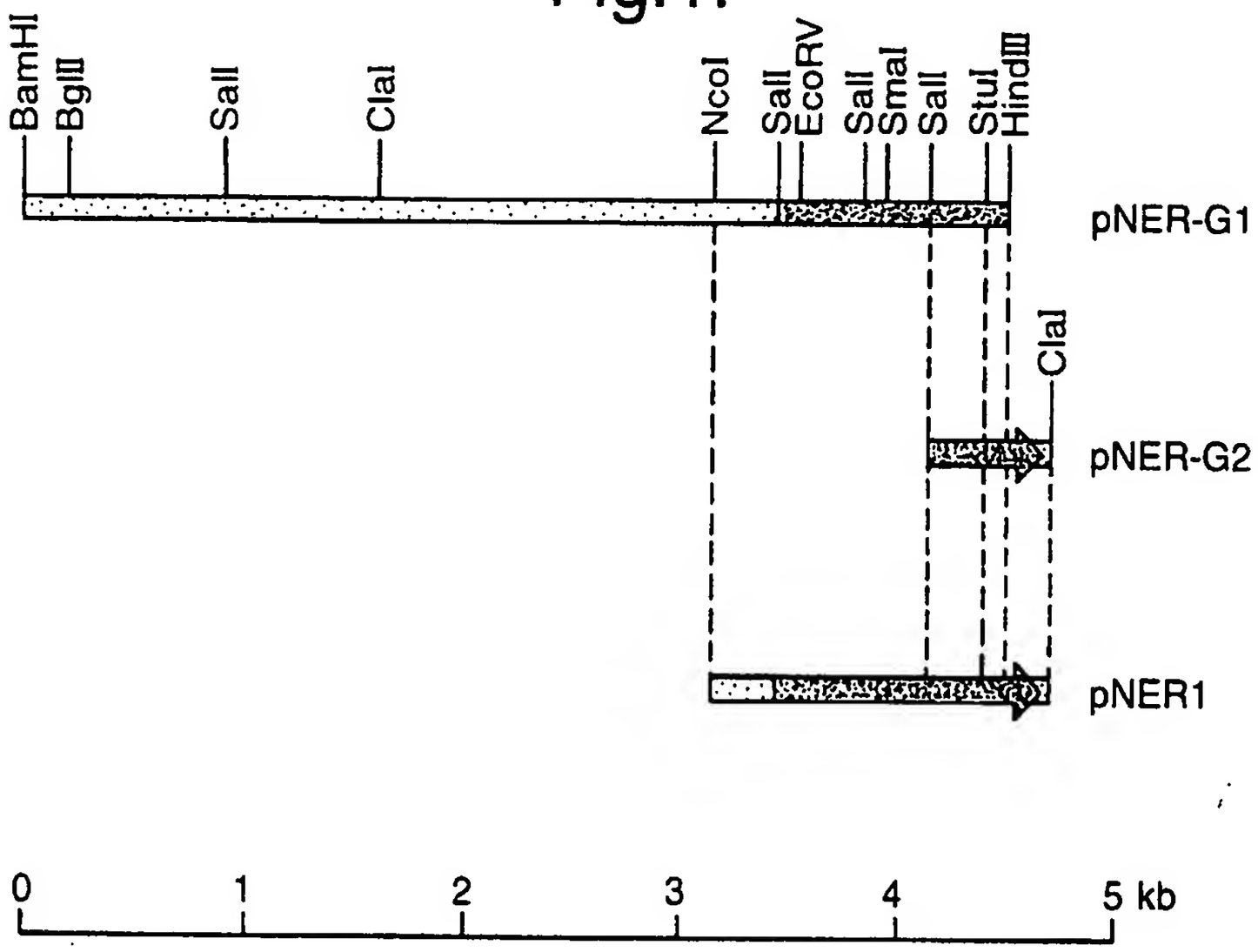

BamHI
BglII
SalI
ClaI
NcoI
SalI
EcoRV
SalI
SmaI
SalI
StuI
HindIII
pNER-G1
ClaI
pNER-G2
pNER1
0
1
2
3
4
5 kb

Fig.2.

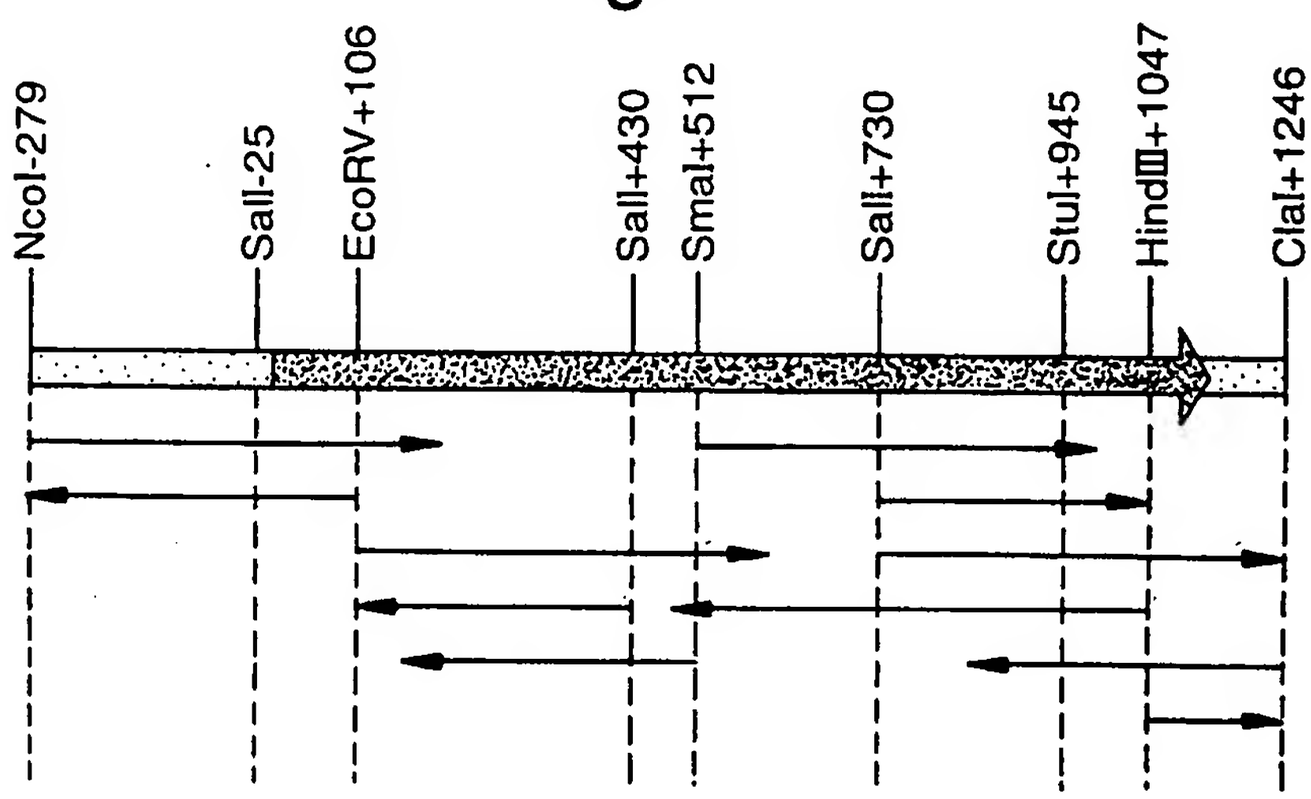

NcoI-279
SalI-25
EcoRV+10
SalI+430
SmaI+512
SalI+730
StuI+945
HindIII+104
ClaI+1246

Fig.3.

NcoI(-279)
ccatggataa aggagccagc ggcgtgattg ccctgttggc tcaggcgctg -230

gagagcgggc gcaatgaaaa aacgctctcc ttctccggcg atccgctcac -180

gcaggcacag gtgctctatt ccctctggtt aggcgccaac ctgcaagcaa -130

aaatgtctcg cagcgccgtg ccgctcgaaa gcgcgctggc gcatgtgaaa -80

aactgtatta ccgcgcctgg cgtgtagccg gcgtttttat ttacccttt -30

SalI(-25) rbs
actagtcgac tggtctactc aggagccgtt atgtccgctg aaaagctgtt +20
M S A E K L F

tacccccactg aaagtgggtg ccgttactgc cccaaaccgc gtgtttatgg +70
T P L K V G A V T A P N R V F M

EcoRV(106)
ccccacttac ccgtctgcgc agcatcgagc cgggcgatat cccaacgcca +120
A P L T R L R S I E P G D I P T P

ttgatgggtg agtattaccg ccagcgcgcc agcgcgggcc tgattatctc +170
L M G E Y Y R Q R A S A G L I I S

cgaagccacg cagatttctg ctcaggcaaa aggctacgcc ggtgcaccgg +220
E A T Q I S A Q A K G Y A G A P

gtctgcacag cccggaacag atcgccgcgt ggaaaaaaat caccgcaggc +270
G L H S P E Q I A A W K K I T A G

gtgcatgctg aagatggccg tattgcggtt cagctgtggc acaccggtcg +320
V H A E D G R I A V Q L W H T G R

tatctcacac agcagcatcc agcctggcgg tcaggcgccg gtttctgcct +370
I S H S S I Q P G G Q A P V S A

ctgccctgaa cgccaatacc cgcacttccc tgcgcgatga aaacggtaat +420
S A L N A N T R T S L R D E N G N

SalI(430)
gcgatccgcg tcgacaccac cacgccacgc gcgctggagc tggacgagat +470
A I R V D T T T P R A L E L D E I

SmaI(471) *SmaI(512)*
cccgggtatc gtgaatgatt tccgtcaggc cgtcgccaac gcccgggaag +520
P G I V N D F R Q A V A N A R E

Fig.3.(Cont.)

BstEII(556)

cgggcttcga cctggttgag cttcactctg cgcacggtta cctgctgcat +570
A G F D L V E L H S A H G Y L L H

cagttcctgt ccccgtcttc caaccagcgt accgaccagt acggcggcag +620
Q F L S P S S N Q R T D Q Y G G S

cgttgaaaac cgcgcgcgtc tggtgcttga agtggtggat gctgtctgta +670
V E N R A R L V L E V V D A V C

atgagtggag cgcagaccgc attggtattc gtgtctcccc gatcggtact +720
N E W S A D R I G I R V S P I G T

SalI(730)

ttccagaacg tcgacaacgg tccgaacgaa gaagcagacg cgctgtatct +770
F Q N V D N G P N E E A D A L Y L

gattgaagag ctggcgaaac gcggtatcgc ctatctgcac atgtccgaga +820
I E E L A K R G I A Y L H M S E

cggacttggc aggcggcaag ccttacagtg aagccttccg tcagaaagtg +870
T D L A G G K P Y S E A F R Q K V

cgcgagcgct tccacggcgt gattatcggg gcgggtgcgt atacggcaga +920
R E R F H G V I I G A G A Y T A E

StuI(945)

aaaagccgag gatttgatcg gtaaaggcct gatcgacgcc gtggcctttg +970
K A E D L I G K G L I D A V A F

gccgtgacta cattgctaac ccggatctgg ttgcccgttt gcagaaaaaa +1020
G R D Y I A N P D L V A R L Q K K

HindIII(1047)

gccgaactga acccgcagcg tcctgaaagc ttctatggcg gcggcgcgga +1070
A E L N P Q R P E S F Y G G G A E

aggttatacc gactaccctt cactgtaatc ccgctttgta cattgatagc +1120
G Y T D Y P S L *

ggcgaccttt cgccgctata ctaaaacatc gtttctgttc aaaaagataa +1170

tccattcgac tggttaatga ggaaattatg cgcctacttc acaccatgct +1220

ClaI(1246)

gcgcgttggc gacctgcaac gttccatcga t

Fig.4.

SAEKLFTPLKVGAVTAPNRVFMAPLTRLRSIEPGDIPTPLM

GEYYRQRASAGLIISEATQISAQAKGYAGAPGLHSPEQIA

AWKKITAGVHAEDGRIAVQLWHTGRISHSSIQPGGQAPVS

ASALNANTRTSLRDENGNAIRVDTTTPRALELDEIPGIVN

DFRQAVANAREAGFDLVELHSAHGYLLHQFLSPSSNQRTD

QYGGSVENRARLVLEVVDAVCNEWSADRIGIRVSPIGTFQ

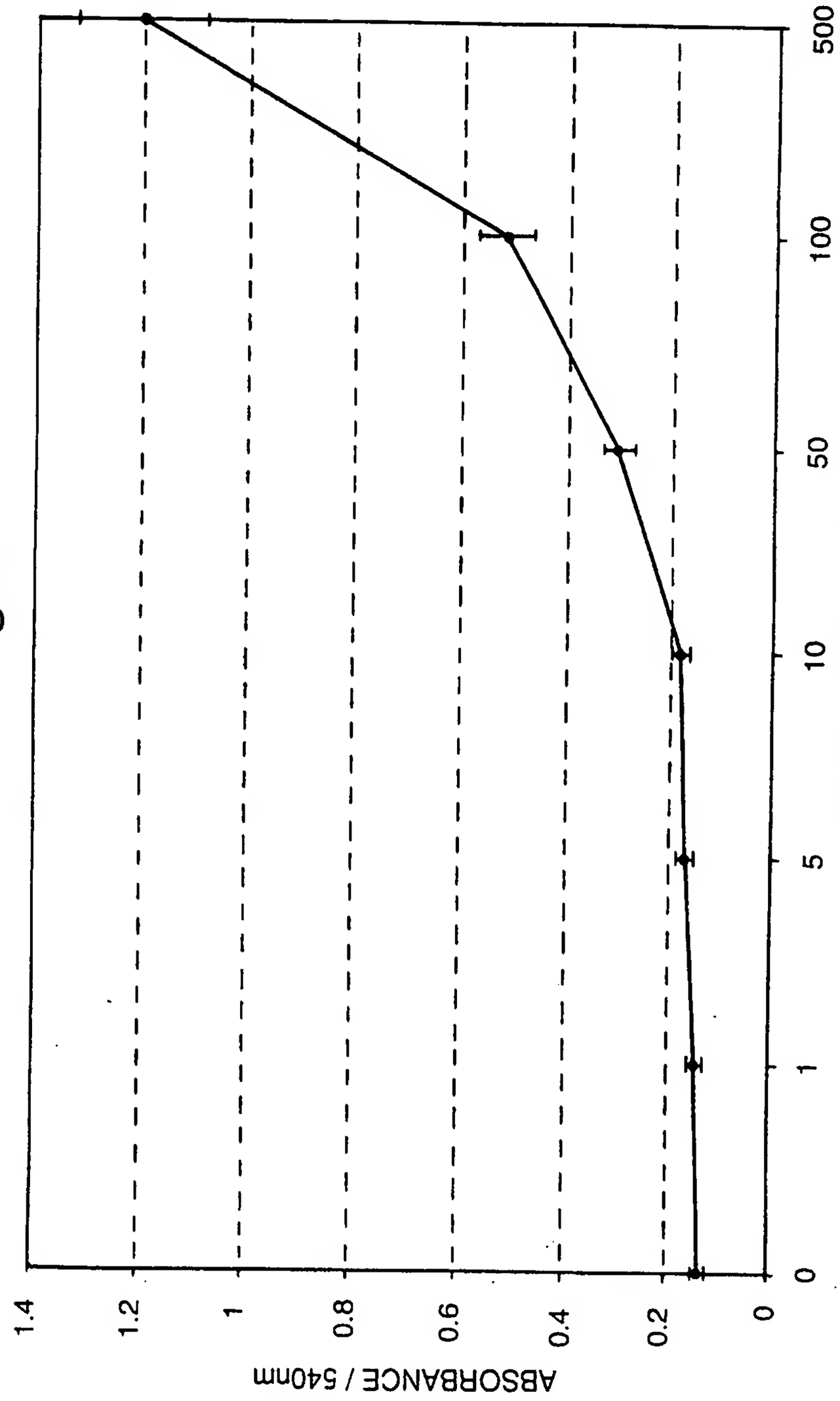
NVDNGPNEEADALYLIEELAKRGIAYLHMSETDLAGGKP

YSEAFRQKVRERFHGVIIGAGAYTAEKAEDLIGKGLIDAV

AFGRDYIANPDLVARLQKKAELNPQRPESFYGGGAEGYT

DYPSL

Fig.5.


ABSORBANCE / 540nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
5
10
50
100
500
AMOUNT PETN/ug/g SOIL

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/01629

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N9/06 C12Q1/00 C12Q1/26 B09C1/10
C12P13/00 C12N1/21 C12N1/20 //(C12N1/20,C12R1:01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N B09C C12P C12R G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 109, no. 7, 15 August 1988 Columbus, Ohio, US; abstract no. 47787, POSADAS DEL RIO, FRANCISCO A. ET AL: "Biotransformation of organic nitrate esters in vitro by human liver, kidney, intestine, and blood serum" XP002018204 see abstract & DRUG METAB. DISPOS. (1988), 16(3), 477-81 CODEN: DMDSAI;ISSN: 0090-9556, ---	1
A	WO,A,94 21394 (UNIV UTAH) 29 September 1994 see claims --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1996	19. 11. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Delanghe, L

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/GB 96/01629

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 511 729 (THERMEDICS INC) 4 November 1992 see claims ---	1
A	EP,A,0 501 353 (TAKEDA CHEMICAL INDUSTRIES LTD) 2 September 1992 see claims ---	1
P,A	WO,A,95 33068 (BRITISH NUCLEAR FUELS PLC ;GARNHAM GEOFFREY WILLIAM (GB)) 7 December 1995 see claims ---	1
P,X	CHEMICAL ABSTRACTS, vol. 125, no. 3, 15 July 1996 Columbus, Ohio, US; abstract no. 27992, BINKS, PETER R. ET AL: "Degradation of pentaerythritol tetranitrate by Enterobacter cloacae PB2" XP002018205 see abstract & APPL. ENVIRON. MICROBIOL. (1996), 62(4), 1214-19 CODEN: AEMIDF;ISSN: 0099-2240, -----	1-3

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 96/01629

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9421394	29-09-94	AU-A-	6490494	11-10-94
EP-A-0511729	04-11-92	CA-A-	2060729	21-09-92
		DE-D-	69210676	20-06-96
		ES-T-	2090493	16-10-96
		US-A-	5476794	19-12-95
EP-A-0501353	02-09-92	JP-A-	5192187	03-08-93
		US-A-	5215919	01-06-93
WO-A-9533068	07-12-95	AU-A-	2677095	21-12-95
		EP-A-	0711357	15-05-96